Hereditas 151: 43–54 (2014)

Multiplexing with three-primer PCR for rapid and economical microsatellite validation

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Vartia, S., Collins, P. C., Cross, T. F., FitzGerald, R. D., Gauthier, D. T., McGinnity, P., Mirimin, L. and Carlsson, J. 2014. Multiplexing with three-primer PCR for rapid and economical microsatellite validation. – *Hereditas* 151: 43–54. Lund, Sweden. eISSN 1601-5223. Received 20 January 2014. Accepted 20 March 2014.

The next generation sequencing revolution has enabled rapid discovery of genetic markers, however, development of fully functioning new markers still requires a long and costly process of marker validation. This study reports a rapid and economical approach for the validation and deployment of polymorphic microsatellite markers obtained from a 454 pyrosequencing library of Atlantic cod, *Gadus morhua,* Linnaeus 1758. Primers were designed from raw reads to amplify specific amplicon size ranges, allowing effective PCR multiplexing. Multiplexing was combined with a three-primer PCR approach using four universal tails to label amplicons with separate fluorochromes. A total of 192 primer pairs were tested, resulting in 73 polymorphic markers. Of these, 55 loci were combined in six multiplex panels each containing between six and eleven markers. Variability of the loci was assessed on *G. morhua* from the Celtic Sea (n = 46) and the Scotian Shelf (n = 46), two locations that have shown genetic differentiation in previous studies. Multilocus F_{ST} between the two samples was estimated at 0.067 (P = 0.001). After three loci potentially under selection were excluded, the global F_{ST} was estimated at 0.043 (P = 0.001). Our technique combines three-primer and multiplex PCR techniques, allowing simultaneous screening and validation of relatively large numbers of microsatellite loci.

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Thirty years after their discovery in the 1980s, microsatellite-based genetic markers are still extensively used in studies of population structure, parentage analysis, genetic mapping, evolutionary processes and forensics (Bruford and Wayne 1993; Brockmann et al. 1994; Knapik et al. 1998; Goldstein et al. 1999; Primmer et al. 2000). These markers have a wide application due to high allelic diversity and co-dominance of alleles (Chambers and MacAvoy 2000). Many methodologies have been established in order to discover such markers, but it is only following the recent advent of next generation sequencing (NGS) technology that large amounts of markers can be increasingly rapidly and economically developed from non-model organisms. NGS approaches allow the fast discovery of large amounts of microsatellite-containing sequences, however mining such data for suitable DNA fragments and validation of candidate markers are still posing challenges prior to the utilisation of fully operating new markers.

The most common approach to date for de novo microsatellite marker development includes creation of repeat-enriched DNA libraries, fragment replication by cloning, and Sanger sequencing of clones containing potential microsatellites (Zane et al. 2002). These processes are laborious and time consuming, and typically have low marker yield, with the percentage of positive clones averaging 2–3% (Ashworth et al. 2004). The final marker yield is even lower with a large portion of markers discarded during the isolation–characterisation process (Squirrell et al. 2003). Alternatively, microsatellite-containing sequences can be mined from existing molecular data such as genomic DNA or expressed sequence tag (EST) sequences (Li et al. 2004). These approaches are limited by the paucity of data on non-model organisms. EST-linked microsatellites can be relatively easy to identify but have a higher probability of being affected by selective processes, and hence may not be suitable for

 DOI: 10.1111/hrd2.00044

population analyses that assume that loci are selectively neutral (Ellis and Burke 2007). Microsatellite markers developed for one species may also be applied to closely related species (Schlötterer et al. 1991). However, this approach is limited by varying levels of successful cross-species amplification between species (Moore et al. 1991). Even when cross-species amplification is successful, levels of variability tend to be lower compared with the species for which the markers were developed (Primmer et al. 1996). Because of these limitations, it may be preferable to develop markers de novo for a species or population of interest to ensure optimal power of newly discovered markers (Carlsson et al. 2013).

Recently, several approaches have been presented for discovery of microsatellites using next generation sequencing (NGS)-generated data (Abdelkrim et al. 2009; Allentoft et al. 2009). Large amounts of sequence data can be generated from either genomic DNA or microsatellite enriched libraries (Guichoux et al. 2011; Malausa et al. 2011) and then mined for microsatellite loci. With this approach, thousands of potential loci can be rapidly identified (Guichoux et al. 2011). Large-scale microsatellite identification has several advantages, including the ability to rigorously screen individual loci for presence of optimal primer-binding sites in flanking regions (Guichoux et al. 2011; Zalapa et al. 2012; Fernandez-Silva et al. 2013). Additionally, deployment criteria (e.g. higher levels of variability, neutrality and low linkage) are study-specific and cannot be assessed until the markers have been validated (Selkoe and Toonen 2006). Validation of a large number of markers enables selection of most suitable loci (Selkoe and Toonen 2006). Even with the NGS approach, validation is labour intensive and a limiting bottleneck in microsatellite marker development (Squirrell et al. 2003; Malausa et al. 2011; Fernandez-Silva et al. 2013).

Typically, methods for validation and genotyping of microsatellites involve capillary gel electrophoresis with fluorescence-based amplicon detection (Edwards et al. 1991; Guichoux et al. 2011). The three-primer PCR method can be used to reduce the expense associated with fluorescently labelled primers (sensu Schuelke 2000; Diniz et al. 2007; Rubin et al. 2009). In three-primer PCR, the primers comprise an unlabelled forward primer with a universal tail attached to its 5′ end, a labelled universal primer matching the tail sequence and an unlabelled reverse primer (Steffens et al. 1993; Oetting et al. 1995; Neilan et al. 1997; Schuelke 2000). The labelled universal primer can be used in combination with any appropriately tailed forward primer, thereby eliminating the need to synthesize a fluorescently labelled forward primer for every unique locus during the validation phase, in which a large proportion of loci may be excluded because of problems with amplification.

Following initial identification and PCR optimisation of successful markers, sets of primers are usually labelled with a fluorescent label either on the forward or the reverse primer (Guichoux et al. 2011). Markers are then amplified in single PCR reactions or combined into a multiplex PCR containing multiple markers (Guichoux et al. 2011). Improvement of the traditional multiplex PCR technique (Missiaggia and Grattapaglia 2006) employed human microsatellite primer sequences as universal tails and combined three universal tails with three dyes in a true multiplex PCR (sensu Guichoux et al. 2011). However, despite the obvious cost benefits of the three primer PCR approach in combination with multiplexing, few studies have employed the method (Langen et al. 2011; Blacket et al. 2012). This may be attributable to poor amplification or poor quality chromatograms, resulting in difficulty in accurate genotyping of individuals (Hagell et al. 2013).

Here we present the development and application of a method for rapid validation and genotyping of novel microsatellites in Atlantic cod, *Gadus morhua,* Linnaeus 1758, using the three primer approach with multiplex PCR. The main aim of this study is the fast and economic development and deployment of microsatellite multiplexes from raw NGS data applicable for studies on a wide range of organisms.

MATERIAL AND METHODS

Sampling

Gadus morhua were obtained by trawling in 2009 and 2011 from the Celtic Sea, south of Ireland (n = 7, n = 46, respectively) and in 1996 from the Scotian Shelf, off Nova Scotia in eastern Canada (n = 46). Previous research has shown that these two populations are genetically differentiated (Hutchinson et al. 2001; O'Leary et al. 2007). Fin clip samples were preserved in 100% ethanol.

DNA extraction

DNA was extracted from fin clips using a Chelex protocol as described in Mirimin et al. (2011). DNA from the Scotian Shelf samples was extracted using a standard phenol–chloroform method (O'Leary et al. 2007). DNA was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific) and normalised to a concentration of 50 ng μl^{-1}.

Microsatellite selection

The unpublished sequence data used here were generated for a previous study (Carlsson et al. 2013), in which microsatellite containing sequences were obtained from five of the 2009 Celtic Sea individuals also used in the

present study, using 454 pyrosequencing of a reduced representation library. Carlsson et al. (2013) identified a total of 11 341 microsatellite containing sequences as suitable for primer design using the Primer3 plug-in (Rozen and Skaletsky 2000) for MISA ver. 1.0 (<http://pgrc.ipk-gatersleben.de/misa>). Of these, 6424 were estimated to be unique. These microsatellite-containing sequences were used in the present study.

To avoid excessive homoplasy (alleles identical in state but not in descent, cf. Estoup et al. 2002) and to ensure ease of genotyping, complex repeat motifs (i.e. compound and imperfect motifs) were excluded. To ensure sufficient space for primer design, reads that had less than 50 bp of sequence before and after the repeat-containing region were removed. In addition, to avoid excessively large allele size ranges, repeat sequences of more than 100 bp, and penta- and hexanucleotide repeats were excluded. A subsample of the remaining microsatellite sequences (n = 1309) were visually inspected for primer design.

Primer design

Primers were designed using Primer3Plus (Rozen and Skaletsky 2000; Untergasser et al. 2007) with optimal primer length as 20bp and optimal T_m at 60°C. Two sets of three size classes were used: the first set of size classes was separated by 30 bp (100–150, 180–250, 280–450 bp), and the second set separated by 50 bp (100–150, 200–250, 300–450 bp). Equal numbers of markers were designed for each size class. Only primer pairs with a T_m difference of less than 1°C were accepted in order to facilitate PCR multiplexing.

Designed primers were cross-referenced with the original sequence data set to identify primers that annealed to multiple regions (not unique) or originated from redundant sequences (different reads of the same sequence). Redundant sequences not detected in the previous steps (due to sequencing error in the primer regions) were identified by performing a de novo assembly with the remaining candidate loci sequences using Geneious ver. 6.1.5 (created by Biomatters; available from <www.geneious.com>), CAP3 plug-in (default settings; i.e. min overlap length = 40 bp, min overlap identity = 90%; Huang and Madan 1999). If two or more reads assembled together they were considered redundant and only one of them was kept for future analysis.

To minimise the risk that primer sequences were derived from contaminants, expressed *G. morhua* gene regions or previously published *G. morhua* microsatellites, microsatellite-containing sequences and primers were subjected to a BLAST search in the GenBank nucleotide database (Altschul et al. 1990). For possible contaminants, we considered a match with ≥ 95% coverage and 100% identity as a threshold for excluding reads. No threshold was employed for matches on *G. morhua* sequences. When such matches were encountered, primers were excluded from further analyses. In addition, validated primer sequences were subjected to BLAST searches against the *G. morhua* genome (Star et al. 2011) in the whole-genome shotgun contigs database in GenBank.

Universal primers

The universal dye-labelled primers used were T3: PET-5′ AATTAACCCTCACTAAAGGG 3′, M13 Reverse: NED-5′ GGATAACAATTTCACACAGG 3’ (Diniz et al. 2007), Hill: 6FAM-5′ TGACCGGCAGCAAAATTG 3′ (Tozaki et al. 2001) and Neomycin rev: VIC-5′ AGGTGAGATGACAGGAGATC 3’. Each forward primer had one of the above universal primer sequences added to its 5′ end. PIG-tails were added to the 5′ end of all the reverse primers. PIG-tailing leads to an addition of a non-templated adenosine nucleotide to the 3′ end on nearly 100% of PCR products which reduces stutter caused by random addition of dATP (Brownstein et al. 1996). The tails were matched with the primers using OligoAnalyzer ver. 3.1 (<www.idtdna.com>) to ensure the least amount of different secondary structures. Equal numbers of primers were paired with each of the four different universal primers.

Microsatellite validation

Primers were combined into twelve multiplex PCR reactions containing 12 markers each (12-plex) and validated using all seven 2009 Celtic Sea individuals. Loci that amplified successfully and showed polymorphism were combined into further multiplexes. The construction of multiplexes was done by means of successive attempts of adding and removing loci from sets of markers that had amplified together in the initial test panels until at least six loci were successfully combined in a panel. When amplified loci were monomorphic, the procedure was repeated on seven Scotian Shelf samples to assess whether they were monomorphic in these individuals.

Multiplex PCRs were performed in 5 µl reactions with 50 ng template DNA, 1 × Multiplex PCR Master Mix (Qiagen), 0.2 µM of each reverse primer, 0.05 µM of each unlabelled forward primer (modified with the appropriate universal tail) and 0.2 µM of labelled universal primer for each forward primer labelled with matching universal tail. Further adjustments made to optimize concentrations of primers in the PCR reactions are given in Table 1. PCR thermal cycling conditions were as follows: 1 × 95°C (15 min); 30 × 94°C (30 s), 60°C (90 s), 72°C (60 s); 8 × 94°C (30 s), 53°C (90 s), 72°C (60 s); 1 × 60°C (30 min). No-template controls were included to monitor for potential contamination.

A total of 1 µl of the multiplex PCR product was added to 9 µl of Super-DI Formamide (MCLAB) with 0.01 µl of Orange DNA Size Standard (MCLAB) and run on an ABI 3130xl Genetic Analyzer according to manufacturer's recommendations. GeneMarker ver. 1.97 (<www.softgenetics.com>) was used for fragment length analysis.

Microsatellite genotyping

All 46 Celtic Sea and 46 Scotian Shelf samples were genotyped with multiplex panels. Genotype data were inspected with Micro-Checker ver. 2.2.3 for genotyping errors and presence of null-alleles (van Oosterhout et al. 2004) using default settings. The 99% confidence interval was used when checking for null alleles to avoid false positives resulting from multiple tests. MSAnalyser ver. 4.05 (Dieringer and Schlötterer 2003), using default settings, was used to assess the number of alleles, allelic richness, allele size ranges, F_{ST} estimates and expected and observed heterozygosity. Data were analysed for possible departure from Hardy–Weinberg equilibrium, linkage disequilibrium, and excess and deficit of heterozygotes using Genepop ver. 4.2 with default settings (Raymond and Rousset 1995; Rousset 2008). False discovery rate (FDR) was used to correct for multiple comparisons (Benjamini and Yekutieli 2001) with initial $\alpha = 0.05$. Lositan (Antao et al. 2008) was used to detect loci that could be under positive or balancing selection (settings "Neutral' mean F_{ST}' and 'Force mean F_{ST}' with 10 000 simulations were used under both the infinite allele model and stepwise mutation model).

F_{ST} replicate sampling

The current study purposefully aimed to validate more markers than required for accurate evaluation of population differentiation (i.e. multilocus F_{ST}). To estimate the number of microsatellite loci future studies on cod population structure may require we investigated how many markers were needed to accurately estimate multilocus F_{ST}. Data sets were generated by randomly drawing 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 loci from the 55-locus dataset with each condition (number of loci) replicated ten times. Average F_{ST} and 95% confidence interval of the ten replicates were calculated and plotted to visualise the variability of average F_{ST} estimates as a function of numbers of markers (Fig. 1).

RESULTS

Primer design

Of 1309 candidate microsatellite loci, 559 were determined to be suitable for primer design upon visual inspection. A total of 349 primer pairs were rejected based on T_m difference, sequence redundancy or secondary structure with the 5′ tail. Another 18 were excluded due to a BLAST match (one match to a *Gadus morhua* microsatellite, nine to a *G. morhua* gene and eight to a possible contaminant). The remaining 192 primer pairs were chosen for validation. Of those, 51 failed to amplify and 45 were excluded due to low scorability. Unambiguous amplification of PCR products in the expected size range was successful in 96 of the 192 markers tested (50%), of which 73 showed polymorphism (38%). The 73 polymorphic loci were used to build multiplex panels. Of these 13 were not included in the final multiplexes because of incompatible size, associated fluorochrome or failure to amplify with the other markers in a panel. As a result 60 markers were combined into six multiplex panels ranging between eight and twelve loci. Five markers were not used in the final analysis due to ambiguous genotyping leading to high chance of scoring errors, resulting in a final panel of 55 polymorphic markers combined into six multiplexes (Table 1). The results of the BLAST search on the validated primers against the *G. morhua* genome are presented in the Supplementary material Appendix 1 Table A1.

Application of markers to test populations

The mean allelic richness (R_S) was 7.1 (SD = 4.11) in the Celtic Sea sample and 7.2 (SD = 4.24) in the Scotian Shelf sample. The minimum number of alleles was two for both the Celtic Sea and Scotian Shelf samples; the maximum number of alleles was 21 and 25, respectively (Supplementary material Appendix 1 Table A2). Micro-Checker analyses indicated no genotyping errors. However, ten loci had a different repeat pattern than the motif originally identified from the raw sequence (Supplementary material Appendix 1 Table A3). Null alleles were observed in 13 loci in the Celtic Sea sample and 11 in the Scotian Shelf sample (Supplementary material Appendix 1 Table A4). Twelve and eleven loci deviated significantly from Hardy–Weinberg equilibrium (after FDR correction) in Celtic Sea and Scotian Shelf samples, respectively. Linkage disequilibrium was observed (after FDR correction) in locus pair A43_T3 x C01_M13 in the Celtic Sea sample; and locus pairs A43_T3 x B19_T3 and C15_Hill x C17_M13 in the Scotian Shelf sample. Lositan identified loci A11_Hill, C40_M13, C42_M13 and D14_Hill as being potentially affected by positive selection, (both under IA and SMM). Only C40_M13 (global $F_{ST} = 0.581$), C42_M13 (global $F_{ST} = 0.301$) and D14_Hill (global $F_{ST} = 0.246$) remained significant after correction for multiple comparisons.

Global multilocus F_{ST} was estimated at 0.067 (P = 0.001). After the three outlier loci identified by Lositan were excluded, the global F_{ST} was estimated at

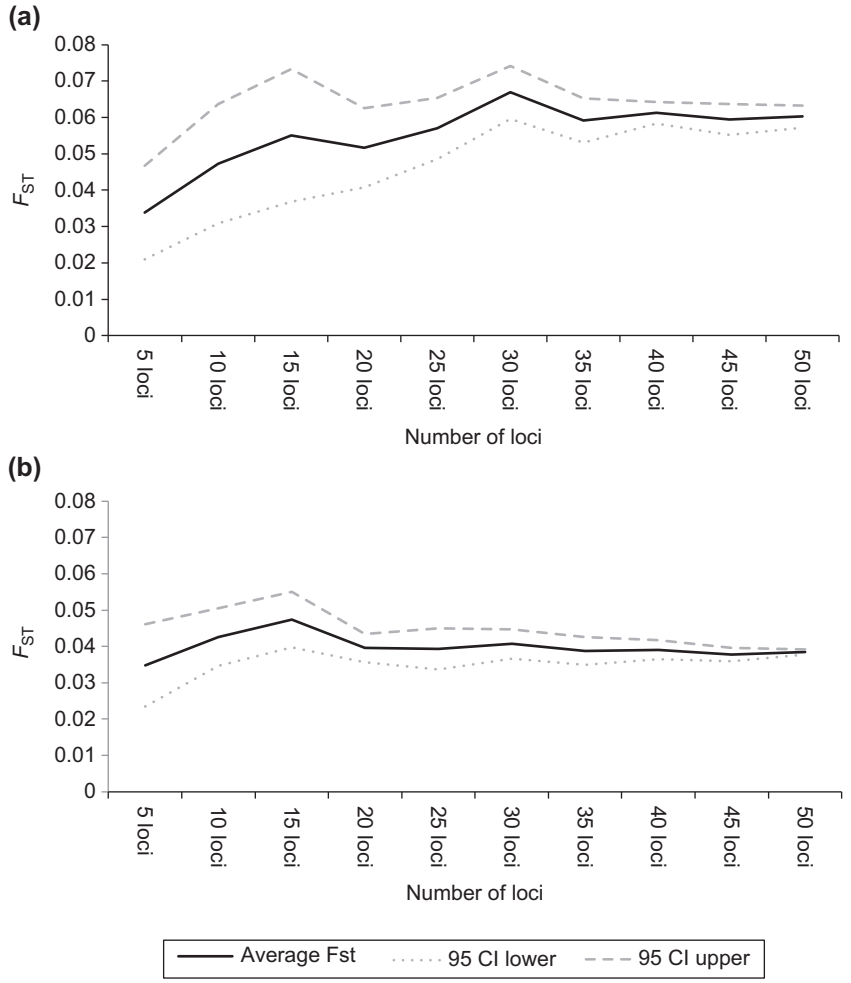

Fig. 1a–b. F_{ST} replicate sampling with all 55 loci (**a**) and with outliers excluded (**b**). 95% confidence interval is displayed.

0.043 (P = 0.001). Global F_{ST} values of individual loci are presented in Supplementary material Appendix 1 Fig. A1.

Replicate sampling of loci to visualise the effect of increasing numbers of loci on F_{ST} estimates and their variances is presented in Fig. 1. This was done in order to see how many loci were needed to reach F_{ST} point estimates with low variances to accurately describe the level of genetic variability between the Celtic Sea and Scotian Shelf samples. The procedure was performed both with and without loci under potential selection. In both cases, increased number of markers reduced the variation in multilocus F_{ST} estimates.

DISCUSSION

Since the initial reports of three-primer PCR (Steffens et al. 1993; Oetting et al. 1995; Neilan et al. 1997; Schuelke 2000) the approach has gained wide acceptance, particularly for initial validation while using conventional two-primer PCR for genotyping (Guichoux et al. 2011; Hunter and Hart 2013; Olafsdottir et al. 2013; Skirnisdottir et al. 2013). Similarly, multiplex amplification of microsatellites is now commonly employed. In a few instances, these two techniques have been combined for microsatellite deployment (Missiaggia and Grattapaglia 2006; Langen et al. 2011; Blacket et al. 2012). However, the combined three primer/multiplex PCR approach, as used here for both microsatellite development and deployment, has not to our knowledge been previously reported. The lack of such studies may reflect conservative views on multiplexing and/or the limited availability of suitable universal primers (Guichoux et al. 2011; Blacket et al. 2012). The three-primer/multiplex PCR approach for validation and genotyping has several characteristics that facilitate cost savings (consumables and labour) relative to other approaches. Fluorescently labelled primers are typically an order of magnitude more expensive than unlabelled primers. Therefore, direct modification of locus specific

Table 1. *Six multiplex panels for amplifying 55 loci. Underlined sequence in the forward primer signifies the universal tail sequence, whereas underlined sequence in the reverse primer signifies the PIG-tail sequence. Adjustment to primer concentration applies for all three primers used to amplify a locus (forward, reverse and universal primer). SRA accession number for the sequences is SRP041380.*

Multiplex	Name	Motif	Size range	Adjustment to primer concentration	Forward primer	Reverse primer
1	A08_T3	tetra	163–187		AATTAACCCTCACTAAAGGGATCTCGAGTGGCGCAGTAG	GTTTCTTGCACGCTGACAAGTGAAGAG
	A16_M13	tetra	156–194		GGATAACAATTTCACACAGGCGTTCCCTCAGCTTGTCTCT	GTTTCTTCGTCCAACAGATCTATGCAATC
	A43_T3	tetra	309–369		AATTAACCCTCACTAAAGGGCCACTTTAACCTGCGGTTTC	GTTTCTTGCCTGCTTGTATACGCTGA
	B19_T3	tri	242–320		AATTAACCCTCACTAAAGGGAAAAGGATCTGCTTG CCTCA	GTTTCTTCGTGAGCTCAGTTTTGGCTA
	B38_Neo	tri	388–415		AGGTGAGATGACAGGAGATCGAATTGAGGAGGC ATGGGTA	GTTTCTTGGTTAATTCCAGCCGTAGAGG
	C15_Hill	tetra	169–205		TGACCGGCAGCAAAATTGCCTTTCGTTCTCTCCGTCAG	GTTTCTTGAGGATTTGGTGGGATGAT
	C28_Neo	tri	255–282		AGGTGAGATGACAGGAGATCCAGCACAAGTGGT AGGGTCA	GTTTCTTGCGATCAGAAGTTGTGCTT
	C36_Hill	tri	319–352		TGACCGGCAGCAAAATTGGTTGGCTCACACAATCATCG	GTTTCTTATCCTTCAAACAGCCCTCAA
	C40_M13	tri	276–277		GGATAACAATTTCACACAGGGGTCTTTGGGAGGTCTTCCT	GTTTCTTCATCCTGCTTGCGGACTTAT
	D14_Hill	tetra	228–276		TGACCGGCAGCAAAATTGGGCGATAATCTGCCATTTTG	GTTTCTTCGTGACAAGTGTCGATTTGC
	D30_M13	tetra	317–333		GGATAACAATTTCACACAGGTTCACAAACGGGAACTACGA	GTTTCTTGACAAGTCCAAGATGTGTCA TCA
2	A18_M13	tetra	203–243		GGATAACAATTTCACACAGGGACTGTCCGTTGAG GGTGTT	GTTTCTTGGGTCGAACTGGTCTGGTTA
	A19_T3	tetra	242–274		AATTAACCCTCACTAAAGGGTCCTGGTTCCAACACATGAC	GTTTCTTCTAGCCAATGGTGCAAGT
	A34_M13	tetra	297–321		GGATAACAATTTCACACAGGTCCTTAACGACAGGCACCTT	GTTTCTTCCTGACTTGTGTCGTTCCAG
	A37_Neo	tetra	265–489		AGGTGAGATGACAGGAGATCCCGGCAGTACAGC TAATGAA	GTTTCTTAAATGCTCAACCCATTGGAC
	B03_T3	di	147–161		AATTAACCCTCACTAAAGGGCGATAATAGCGTTCCCATCC	GTTTCTTGGGGTACCTTGTGACCTGT
	B12_T3	di	346–398	2x	AATTAACCCTCACTAAAGGGGCTTTGGCAACACTGTTTGA	GTTTCTTGTCGAGCAGACCAGAAGACC
	B30_Neo	tri	151–169		AGGTGAGATGACAGGAGATCTTGTACGGACAGG AAGTCCA	GTTTCTTGCGAACAGTGTGTAAATTGAA
	C01_M13	tetra	115–195		GGATAACAATTTCACACAGGACCAGGAGGTTGGATCAGTG	GTTTCTTCCATTATTCATTCGTCATCCA
	C14_Hill	tetra	146–166		TGACCGGCAGCAAAATTGAGGTTCAGCCAGAAGCTGAT	GTTTCTTCCATTGGTTGTCGGTGATTA
	C20_Neo	tri	227–239		AGGTGAGATGACAGGAGATCCCGCCTATCACCCTA AATCTG	GTTTCTTCGTCTACATGTCGTGGTAGGG
	D12_Hill	tetra	251–291		TGACCGGCAGCAAAATTGAACGGCTCCTCAAGACAAAC	GTTTCTTAGGCATCTGCGTCCATACTC
3	A33_M13	tetra	233–297		GGATAACAATTTCACACAGGAGACACTGAGCTC GACAGCA	GTTTCTTATCAATGATCCCAGGCAAAC
	A39_Hill	tetra	356–416		TGACCGGCAGCAAAATTGCCTGTCCAAATGCACACAAG	GTTTCTTAGTGCTTGGATGGTGTGATG
	B01_Hill	di	129–213		TGACCGGCAGCAAAATTGTAGACTCTGGGGCTGGGTAA	GTTTCTTGCATCCGAGACTCTTGTTCC
	B15_T3	di	170–178		AATTAACCCTCACTAAAGGGCGATGCGATTCTT GGTAAATG	GTTTCTTGGTGTCTCATCCCTCTTCA

Continued

Table 1. *Continued*.

Multiplex	Name	Motif	Size range	Adjustment to primer concentration	Forward primer	Reverse primer
	B29_Neo	tri	262–281		AGGTGAGATGACAGGAGATCGGGAAAGAGCCGGA AAAGTA	GTTTCTTGCTAATGTTGGCAGAACCA
	C13_Neo	tri	158–176		AGGTGAGATGACAGGAGATCGGGTGATTGAGGTTGCGATA	GTTTCTTGCTCACACATCCTACGAGCA
	C17_M13	tetra	154–190		GGATAACAATTTCACACAGGCTTCTCGATGGCATGTTTCC	GTTTCTTCCTGCACAATGATCTGCAT
	C22_Hill	tetra	225–273		TGACCGGCAGCAAAATTGGGCTTGTCGTTGGTTCCTT	GTTTCTTGTTGAATGCAACCCCTCAGT
	C30_Neo	tri	337–343		AGGTGAGATGACAGGAGATCAGGTGGTCGCAGTGA AGAAG	GTTTCTTGGGTGAATGCCTCTTAATCG
	C42_M13	tri	345–358		GGATAACAATTTCACACAGGGCTGAGGGGATGC GATAATA	GTTTCTTAGCCAAGGGTGAAGTGTTGT
	D37_T3	tetra	313–333		AATTAACCCTCACTAAAGGGATGTGACACCGAATCACAGC	GTTTCTTACCCGTCCTGTACGTGAACT
4	A11_Hill	tetra	163–175		TGACCGGCAGCAAAATTGCGACAGGGAGGCA TAAAGAC	GTTTCTTGTTCACCTCCCTGGCTCTT
	A22_Neo	tetra	214–243		AGGTGAGATGACAGGAGATCGGTGAGGTTCTT GAGGGTCA	GTTTCTTGATTATTTCCCCCTGCTG
	A31_T3	tetra	314–386		AATTAACCCTCACTAAAGGGGGATATGTGGGG ATGAGCAC	GTTTCTTATGGGTCCTTCTCCTTTGGT
	B33_M13	tri	120–135	0.5x	GGATAACAATTTCACACAGGCTACAGCAGGGG TTCCTCAG	GTTTCTTGTTTGTTGCTCCGATGGACT
	C08_T3	tetra	145–185		AATTAACCCTCACTAAAGGGCTCGGACCCAGA GATCAAAA	GTTTCTTGCAGCATCTGAACTGAAACG
	D15_Hill	tetra	244–256		TGACCGGCAGCAAAATTGTGACTCAACGGAGGTACGTG	GTTTCTTCCATCAGGATCAGGACCACT
	D21_Neo	tetra	337–389		AGGTGAGATGACAGGAGATCAACACGCTTGCT GGGACTAC	GTTTCTTCACTGGAGTGTACGGTCTCTGA
	D46_Neo	tetra	139–155		AGGTGAGATGACAGGAGATCCCTCCCTAATACCA TGTCACCA	GTTTCTTCGTCTGTTCACGGATGCAC
5	A04_Neo	tetra	143–211		AGGTGAGATGACAGGAGATCACAATCAACCCTCCAACTCG	GTTTCTTCAGGTCCCGAATATCAAGG
	B07_M13	di	163–187		GGATAACAATTTCACACAGGTGGACAATTACATTGAAA ATCACAG	GTTTCTTCCTGAACTGCCTGTCAATG
	B28_Neo	tri	226–238		AGGTGAGATGACAGGAGATCCCCACCCCTTAATGTTTCAA	GTTTCTTGGCGTCATTCTCTTTGATG
	D10_Neo	tetra	308–312	2x	AGGTGAGATGACAGGAGATCCGCCAATGCAAATCTCTTTT	GTTTCTTATCTGAGTGCGTCGAGTGC
	D35_M13	tetra	316–360		GGATAACAATTTCACACAGGTCCACACTTGGTCGATGAAA	GTTTCTTGACGAGTGTCAGCAGGTGTG
	D43_T3	tetra	260–292	2x	AATTAACCCTCACTAAAGGGGTGCCGCTCACGCTACTAAT	GTTTCTTCGTGATCGCTCTCGATTC
6	A03_Hill	tetra	158–170		TGACCGGCAGCAAAATTGGAGCGTGTTGAACGACTTGA	GTTTCTTCCTGAGCAGTGGAGTGACAA
	A20_Neo	tetra	164–182		AGGTGAGATGACAGGAGATCCGAGGCTACACAGCCTGTAA	GTTTCTTACTGTGGGCATGTAACAGCA
	A30_T3	tetra	245–269		AATTAACCCTCACTAAAGGGAGTTGAACTGCGGGTTCTGT	GTTTCTTGCACGATGTCACAGCTGATT
	B36_Hill	tri	384–396		TGACCGGCAGCAAAATTGCCCGCCCAGACATAATAAGA	GTTTCTTCAGTCTCAGCCTCCACATCA
	C31_Neo	tri	332–341		AGGTGAGATGACAGGAGATCGCCAAGACAAGCATTTCCAT	GTTTCTTCGAGCCAGCGTTTACTTCTC
	C35_M13	tri	233–348		GGATAACAATTTCACACAGGGGCAATGTCGTACACCTCAA	GTTTCTTCTGAACGGCAACACTTCGTA
	D05_Hill	tetra	230–270		TGACCGGCAGCAAAATTGACTGCCCCTGATAACAATGC	GTTTCTTAGGCATCGACCATTTGTAGC
	D16_T3	tetra	424–436		AATTAACCCTCACTAAAGGGCCAGCAGCTTTCTGGGTAGT	GTTTCGAAGCGTTACTGCAGACAG

primers substantially increases project costs, especially when markers must be excluded due to low scorability and/or bias (Selkoe and Toonen 2006). The use of fluorescently labelled universal primers avoids these potential complications and further decreases project costs, as a limited number of these primers can be purchased at large synthesis scales. Further multiplexing six to eleven amplicons per ABI capillary lane reduces PCR and genotyping costs as well as labour effort.

Primers were designed to amplify loci in three non-overlapping allele size ranges per dye as reported by Neff et al. (2000), however, larger size separations between ranges were employed. Overlapping size ranges have a disadvantage in that only one marker can be used per dye (Miller et al. 2013a, 2013b). Because actual allele sizes were not known in advance of capillary separation, two gap sizes (30 bp and 50 bp) between marker class size ranges were used to minimize overlap chances within a dye set. In practice, only a single overlap between markers was observed in the combined set of gap sizes, while the remaining markers were separated by at least 8 bp. However, microsatellites generated using the 50 bp gap size were more easily combined in multiplex PCR. We therefore recommend that marker size classes are set apart by at least 50 bp during primer design (e.g. 100–150, 200–250, 300–450 bp) to facilitate combining loci in multiplexes.

Both raw reads and contigs have been used with similar success for microsatellite discovery (Table 2). Contigs can yield more robust primers because increased sequencing depth can be used to detect sequencing errors or genetic variation in the primer binding region (Fernandez-Silva et al. 2013; Zalapa et al. 2012). However, repeat-containing reads may fail to assemble during contig construction, preventing discovery of some valid microsatellite loci (sensu Cavagnaro et al. 2010). Also, if the assembler is not able to distinguish the repeat and uses it as the basis for alignment, the unique flanking regions can easily be erroneously collapsed (Treangen and Salzberg 2012). We used raw reads to maximise microsatellite yield in this study and were able to achieve a 50% amplification success rate for trialled primers.

The design of a multiplex panel usually starts with evaluation of loci in single locus PCR reactions (Neff et al. 2000; Guichoux et al. 2011). For this study, evaluating 192 loci in single PCR reactions would have required 192 additional PCRs on the validation panel of seven individuals and a negative control, and the analyses of the resulting 1536 amplicons via capillary electrophoresis. The elimination of this step reduced primer validation

Table 2. *Comparison of previous studies using either contigs or raw reads in microsatellite discovery via 454 pyrosequencing. The studies employed 454 GS-FLX Titanium chemistry, apart from the publications marked with *which used the 454 GS-FLX chemistry.*

Data	Taxon name	Primers screened	Polymorphic	Primer-to-polymorphic marker proportion	Reference		
Contig	*Neophoca cinerea*	28	12	0.43	Ahonen et al. 2013	Average	0.45
	Cyanoramphus malherbi	35	18	0.51	Andrews et al. 2013*	Median	0.43
	Catha edulis	63	27	0.43	Curto et al. 2013*		
	Stylissa carteri	96	12	0.13	Giles et al. 2013		
	Python molurus bivittatus	26	18	0.69	Hunter and Hart 2013		
	Popenaias popeii	28	20	0.71	Inoue et al. 2013		
	Isoodon obesulus	46	9	0.20	Li et al. 2013		
	Antilocapra americana sonoriensis	100	14	0.14	Munguia-Vega et al. 2013		
	Scomber scombrus	80	30	0.38	Olafsdottir et al. 2013		
	multiple species	16–81	8–25	0.15–0.88	Schoebel et al. 2013		
	Unio crassus	77	11	0.14	Sell et al. 2013*		
	Cyclopterus lumpus	48	22	0.46	Skirnisdottir et al. 2013		
	Kunzea pulchella	27	10	0.37	Tapper et al. 2013		
Raw	*Gadus morhua*	15	6	0.40	Carlsson et al. 2013	Average	0.44
	Mulloidichthys flavolineatus	24	23	0.96	Fernandez-Silva et al. 2013	Median	0.38
	Pleuromamma xiphias	15	8	0.53	Fernandez-Silva et al. 2013		
	Brachyptera braueri	30	5	0.17	Geismar and Nowak 2013		
	Euastacus bispinosus	40	15	0.38	Miller et al. 2013a*		
	Neophema chrysogaster	55	14	0.25	Miller et al. 2013b*		
	Prionace glauca	100	12	0.12	Taguchi et al. 2013		
	Silurus asotus	70	47	0.67	Xu et al. 2013		

time and lowered consumable and labour costs. The present approach will yield markers for use in multiplex panels. However we recognise that some markers that would amplify in single locus PCR may fail in multiplex, therefore potentially lowering the conversion proportion from tested loci to polymorphic loci. Nevertheless, we contend that the increase in speed outweighs the possible loss of potential markers.

The conversion proportion from tested loci to polymorphic loci in this study was 38% (73/192). This value was similar to the conversion proportion of 40% (6/15) observed in the initial small scale validation by Carlsson et al. (2013) using the same data, and is consistent with recent studies using raw reads from 454 GS-FLX sequencing of genomic DNA (Table 2). Considerable variability of primer-to-polymorphic marker proportion has been observed among studies (Table 2). A portion of this variation can be attributed to differences in the genome composition of the study organisms (Schoebel et al. 2013), for example, PCR amplification success is lower in organisms with comparatively large genomes (Garner 2002; Schoebel et al. 2013). This can be due to larger genomes typically harbouring more repetitive elements (Hancock 2002). Microsatellite discovery strategies, such as differences in search parameters and algorithms, or using contigs versus raw sequence reads, can possibly affect the conversion from tested loci to polymorphic loci as well. Variation is also likely caused by different strategies in selection of loci to be validated (Fernandez-Silva et al. 2013).

Implementation in G. morhua

As a proof of concept we applied the described approach to samples of *G. morhua* from the Celtic Sea and the Scotian Shelf. *G. morhua* from the Celtic Sea were used for initial microsatellite development (Carlsson et al. 2013) and the Scotian Shelf *G. morhua* form a genetically distinct population from eastern Atlantic *G. morhua* (Hutchinson et al. 2001; O'Leary et al. 2007). The present study estimated F_{ST} between Celtic Sea and Scotian Shelf *G. morhua* at 0.067 when 55 loci were employed. After exclusion of three loci that were potentially under positive selection, F_{ST} was estimated at 0.043. The reduction in F_{ST} is consistent with previous studies that have demonstrated that inclusion of outlier loci that are potentially under selection can markedly affect F_{ST} estimates (Nielsen et al. 2006; Allendorf et al. 2010). The presence and scale of population structure between Celtic Sea and Scotian Shelf *G. morhua* in the present study concurs with previous studies that examined these populations (Hutchinson et al. 2001; O'Leary et al. 2006, 2007; Pampoulie et al. 2008), and with additional studies that demonstrated population differentiation between the eastern and western Atlantic *G. morhua* (i.e. allozymes, Mork et al. 1985; minisatellite, Galvin et al. 1995; nuclear RFLPs, Pogson et al. 1995, 2001; microsatellites, Bentzen et al. 1996; Hutchinson et al. 2001; O'Leary et al. 2007; Pampoulie et al. 2008; single nucleotide polymorphisms, O'Leary et al. 2006; Nielsen et al. 2009; Bradbury et al. 2010).

The combined microsatellite validation and genotyping approach presented here was designed to be a fast and cost-effective means for developing and deploying large numbers of microsatellite markers. Using larger numbers of genetic markers confers considerable advantages of increased precision and statistical power when assessing intra- and inter-population genetic parameters such as population structure and gene flow, as well as when inferring demographic parameters, such as effective population size, population expansions and bottlenecks (Nei and Tajima 1981; Ryman et al. 2006). This improved precision allows for more robust and trustworthy management advice based on genetic data. In the present case, the rate of reduction of multilocus F_{ST} variability decreased after 20–30 loci suggesting that this is the point where using more loci only slightly improves the precision of the multilocus F_{ST} estimate. The point of diminishing returns may not be the same for other populations, other geographic scales or other organisms. The advantage of the method presented here is that more loci can be effectively genotyped, ensuring that the point of diminishing returns has in fact been reached and the most precise estimate of population genetic parameter acquired.

Conclusions

The current study combines three-primer PCR with multiplexing to allow for more economical, rapid development and deployment of microsatellite markers discovered from high throughput sequencing data. Fifty-five polymorphic *G. morhua* microsatellites were combined into six PCR multiplexes, which allowed for determination of F_{ST} between two populations with high precision. This approach is transferable to any species, including those for which extensive sequence resources are not available, and will allow for large and robust population genetic studies while minimising expensive and labour intensive capillary sequencing runs.

Acknowledgements – This work was supported by the Sea Change Strategy with the support of the Marine Institute and the Marine Research Sub-programme of the National Development Plan 2007-2013, co-financed by the European Regional Development Fund (Grant-aid agreement no. PBA/AF/07/004, EIRCOD). PM and JC acknowledge funding from the Beaufort Marine Research Award in Fish Population Genetics funded by the Irish Government under the Sea Change Programme. The authors would like to acknowledge the staff at Carna Research Station who acquired the Celtic Sea *G. morhua* samples. Alice

Antoniacomi and Jeanette Carlsson are thanked for technical support.

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Supplementary material (available online as Appendix hrd.00044 at <www.hereditasjournal.org/readers/appendix>). Appendix 1.

Hereditas

HRD-00044

Vartia, S., Collins, P. C., Cross, T. F., FitzGerald, R. D., Gauthier, D. T., McGinnity, P., Mirimin, L and Carlsson, J. 2014. Multiplexing with three-primer PCR for rapid and economical microsatellite validation. – *Hereditas* doi: 10.1111/hrd.00044.

Appendix 1

Figure A1. Global F_{ST} –values of individual loci. Unfilled squares signify the loci identified as potentially affected by positive selection.

0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
F_{ST}
D10_Neo
A19_T3
C20_Neo
D37_T3
D15_Hill
C13_Neo
A31_T3
A39_Hill
A34_M13
A22_Neo
B07_M13
B33_M13
C28_Neo
A03_Hill
B12_T3
D16_T3
B15_T3
B30_Neo
C36_Hill
B19_T3
C30_Neo
A37_Neo
A30_T3
B03_T3
C22_Hill
B28_Neo
C14_Hill
A04_Neo
A08_T3
B01_Hill
C15_Hill
A33_M13
D21_Neo
D05_Hill
D30_M13
D35_M13
D46_Neo
C31_Neo
A18_M13
C35_M13
A43_T3
C17_M13
D43_T3
B29_Neo
C01_M13
A16_M13
C08_T3
B36_Hill
A20_Neo
D12_Hill
B38_Neo
D14_Hill
C42_M13
A11_Hill
C40_M13

Table A1. BLAST hits of the 55 microsatellite loci primers searched against Gadus morhua in the Whole-genome shotgun contigs database in Genbank.

Primer name	Name of best hit	Accession no.	% Identity	Length of align.	Query start	Query end	Subject start	Subject end	E value
A03_F	contig236159	CAEA01127894.1	100	20	1	20	450	469	0.006
A04_F	contig561296	CAEA01381837.1	100	20	1	20	93	74	0.006
A08_F	contig329249	CAEA01195481.1	100	19	1	19	906	888	0.023
A11_F	contig547549	CAEA01373975.1	100	20	1	20	1368	1349	0.006
A16_F	contig91315	CAEA01547591.1	95	20	1	20	725	706	0.29
A18_F	contig116377	CAEA01022512.1	100	20	1	20	703	684	0.006
A19_F	contig696497	CAEA01457403.1	100	20	1	20	827	808	0.006
A20_F	contig882913	CAEA01534067.1	100	20	1	20	1552	1571	0.006
A22_F	contig264103	CAEA01146189.1	100	20	1	20	1024	1005	0.006
A30_F	contig179262	CAEA01083664.1	100	20	1	20	561	580	0.006
A31_F	contig889085	CAEA01540758.1	100	20	1	20	7068	7087	0.006
A33_F	contig96246	CAEA01551785.1	100	20	1	20	2499	2480	0.006
A34_F	contig306704	CAEA01177506.1	100	20	1	20	7224	7243	0.006
A37_F	contig406243	CAEA01258542.1	100	20	1	20	1194	1213	0.006
A39_F	contig350193	CAEA01212575.1	100	20	1	20	2369	2350	0.006
A43_F	contig58807	CAEA01397255.1	100	20	1	20	1659	1640	0.006
B01_F	contig80676	CAEA01508858.1	100	20	1	20	1292	1273	0.006
B03_F	contig320571	CAEA01188503.1	100	20	1	20	260	279	0.006
B07_F	contig884319	CAEA01535585.1	100	25	1	25	2639	2663	1.00E-05
B12_F	contig353573	CAEA01215347.1	100	20	1	20	1544	1525	0.006
B15_F	contig55910	CAEA01380961.1	100	21	1	21	4688	4668	0.002
B19_F	contig884794	CAEA01536106.1	100	20	1	20	344	325	0.006
B28_F	contig96405	CAEA01551885.1	95.24	21	1	20	3119	3139	0.29
B29_F	contig111672	CAEA01017946.1	95.24	21	1	20	961	941	0.29

B30_F	contig372227	CAEA01230488.1	100	20	1	20	317	298	0.006
B33_F	contig182835	CAEA01087150.1	100	20	1	20	1474	1493	0.006
B36_F	contig368008	CAEA01227052.1	100	18	1	18	321	338	0.081
B38_F	contig886567	CAEA01538036.1	95	20	1	20	4860	4878	1.1
C01_F	contig889944	CAEA01541680.1	95	20	1	20	1340	1321	0.29
C08_F	contig138840	CAEA01044355.1	100	20	1	20	577	596	0.006
C13_F	contig94964	CAEA01550699.1	100	20	1	20	1313	1332	0.006
C14_F	contig890234	CAEA01541994.1	100	20	1	20	5445	5464	0.006
C15_F	contig283435	CAEA01159677.1	100	20	1	20	61	42	0.006
C17_F	contig885749	CAEA01537152.1	100	20	1	20	627	646	0.006
C20_F	contig119710	CAEA01025720.1	100	21	1	21	1105	1125	0.002
C22_F	contig749929	CAEA01486978.1	100	19	1	19	120	102	0.023
C28_F	contig884951	CAEA01536277.1	100	20	1	20	1372	1391	0.006
C30_F	contig344699	CAEA01208094.1	100	20	1	20	105	124	0.006
C31_F	contig85706	CAEA01525561.1	100	20	1	20	1396	1377	0.006
C35_F	contig549938	CAEA01375710.1	100	20	1	20	3223	3242	0.006
C36_F	contig320962	CAEA01188840.1	100	20	1	20	1940	1921	0.006
C40_F	contig888314	CAEA01539930.1	100	20	1	20	4274	4293	0.006
C42_F	contig40710	CAEA01259242.1	100	20	1	20	6252	6233	0.006
D05_F	contig881311	CAEA01532322.1	100	20	1	20	6332	6313	0.006
D10_F	contig114745	CAEA01020896.1	100	20	1	20	2444	2425	0.006
D12_F	contig882640	CAEA01533770.1	100	20	1	20	4430	4411	0.006
D14_F	contig52321	CAEA01360772.1	100	20	1	20	1098	1079	0.006
D15_F	contig66409	CAEA01438378.1	100	20	1	20	2496	2515	0.006
D16_F	contig880291	CAEA01531221.1	100	20	1	20	1418	1437	0.006
D21_F	contig125767	CAEA01031570.1	100	20	1	20	956	975	0.006
D30_F	contig51563	CAEA01354798.1	100	20	1	20	936	955	0.006
D35_F	contig674890	CAEA01445464.1	100	20	1	20	123	104	0.006
D37_F	contig323213	CAEA01190654.1	100	20	1	20	8387	8406	0.006
D43_F	contig889679	CAEA01541394.1	100	20	1	20	2993	2974	0.006

D46_F	contig296077	CAEA01168917.1	100	22	1	22	344	365	2.00E-04
A03_R	contig73589	CAEA01477685.1	100	16	2	17	1732	1747	1.1
A04_R	contig351852	CAEA01213927.1	100	15	5	19	25	11	3.8
A08_R	contig329249	CAEA01195481.1	100	20	1	20	766	785	0.006
A11_R	contig547549	CAEA01373975.1	100	19	1	19	1227	1245	0.023
A16_R	contig91315	CAEA01547591.1	100	22	1	22	561	582	5.00E-04
A18_R	contig116377	CAEA01022512.1	95	20	1	20	521	540	0.29
A19_R	contig881235	CAEA01532242.1	100	16	1	16	2067	2082	1.1
A20_R	contig125694	CAEA01031500.1	100	18	2	19	277	260	0.081
A22_R	contig264103	CAEA01146189.1	100	20	1	20	818	837	0.006
A30_R	contig891916	CAEA01543820.1	100	17	3	19	4927	4943	0.29
A31_R	contig889085	CAEA01540758.1	100	20	1	20	7402	7383	0.006
A33_R	contig96246	CAEA01551785.1	100	20	1	20	2265	2284	0.006
A34_R	contig306704	CAEA01177506.1	100	20	1	20	7501	7482	0.006
A37_R	contig406243	CAEA01258542.1	100	20	1	20	1472	1453	0.006
A39_R	contig350193	CAEA01212575.1	100	20	1	20	1995	2014	0.006
A43_R	contig58807	CAEA01397255.1	100	20	1	20	1347	1366	0.006
B01_R	contig569386	CAEA01385913.1	100	20	1	20	3059	3040	0.006
B03_R	contig320571	CAEA01188503.1	100	20	1	20	380	361	0.006
B07_R	contig884319	CAEA01535585.1	100	20	1	20	2780	2761	0.006
B12_R	contig889720	CAEA01541440.1	100	16	5	20	767	782	1.1
B15_R	contig55910	CAEA01380961.1	100	20	1	20	4540	4559	0.006
B19_R	contig884794	CAEA01536106.1	100	20	1	20	116	135	0.006
B28_R	contig96405	CAEA01551885.1	100	20	1	20	3324	3305	0.006
B29_R	contig111672	CAEA01017946.1	100	20	1	20	718	737	0.006
B30_R	contig372227	CAEA01230488.1	100	22	1	22	185	206	5.00E-04
B33_R	contig182835	CAEA01087150.1	100	20	1	20	1575	1556	0.006
B36_R	contig364049	CAEA01223793.1	95	20	1	20	151	132	0.29
B38_R	contig886567	CAEA01538036.1	95.24	21	1	21	5245	5225	0.081
C01_R	contig34310	CAEA01206763.1	95.24	21	2	22	2912	2892	0.081

C08_R	contig138840	CAEA01044355.1	100	20	1	20	698	679	0.006
C13_R	contig94964	CAEA01550699.1	100	20	1	20	1449	1430	0.006
C14_R	contig890234	CAEA01541994.1	100	20	1	20	5575	5556	0.006
C15_R	contig753484	CAEA01489240.1	100	20	1	20	86	67	0.006
C17_R	contig885749	CAEA01537152.1	100	20	1	20	771	752	0.006
C20_R	contig344009	CAEA01207549.1	100	17	5	21	2101	2117	0.29
C22_R	contig291538	CAEA01165662.1	95.24	21	1	20	154	174	0.29
C28_R	contig884951	CAEA01536277.1	100	20	1	20	1613	1594	0.006
C30_R	contig344699	CAEA01208094.1	100	20	1	20	410	391	0.006
C31_R	contig538450	CAEA01370120.1	100	15	2	16	360	346	3.8
C35_R	contig549938	CAEA01375710.1	95	20	1	20	3471	3453	1.1
C36_R	contig320962	CAEA01188840.1	95	20	1	20	1639	1658	0.29
C40_R	ontig888314	CAEA01539930.1	100	18	1	18	4523	4506	0.081
C42_R	contig40710	CAEA01259242.1	100	20	1	20	5933	5952	0.006
D05_R	contig881311	CAEA01532322.1	100	20	1	20	6119	6138	0.006
D10_R	contig884493	CAEA01535777.1	94.74	19	1	19	3521	3539	1.1
D12_R	contig882640	CAEA01533770.1	100	20	1	20	4193	4212	0.006
D14_R	contig52321	CAEA01360772.1	100	18	3	20	897	914	0.081
D15_R	contig66409	CAEA01438378.1	100	20	1	20	2716	2697	0.006
D16_R	contig05146	CAEA01003665.1	100	16	2	17	5870	5885	1.1
D21_R	contig88561	CAEA01536999.1	95.45	22	1	22	1515	1535	0.081
D30_R	contig51563	CAEA01354798.1	95.83	24	1	23	1235	1212	0.006
D35_R	contig391096	CAEA01246040.1	100	17	2	18	511	495	0.29
D37_R	contig323213	CAEA01190654.1	95	20	1	20	8685	8667	1.1
D43_R	contig433731	CAEA01281599.1	94.44	18	1	18	3128	3145	3.8
D46_R	contig296077	CAEA01168917.1	100	19	1	19	466	448	0.023

Table A2. Summary statistics for 55 microsatellite loci in two samples of Atlantic cod. n, number of individuals; a, number of alleles; R_S, allelic richness per locus and sample; as, allele size range in base pairs; H_E, expected heterozygosity; H_O, observed heterozygosity; HW, probability values of concordance with Hardy–Weinberg expectations. Values in bold type are significant probability estimates after false discovery rate (FDR) correction for multiple tests (α = 0.05).

	Celtic Sea							Scotian Shelf						
Locus name	*n*	*a*	R_S	*as*	H_E	H_O	HW	*n*	*a*	R_S	*as*	H_E	H_O	HW
A03_Hill	45	4	4.00	158–170	0.687	0.733	0.885	46	4	4.00	158–170	0.586	0.652	0.217
A04_Neo	45	16	16.00	143–203	0.903	**0.711**	**0.004**	46	18	17.91	143–211	0.925	**0.674**	**0.000**
A08_T3	46	6	6.00	163–183	0.698	0.630	0.187	46	7	7.00	163–187	0.569	0.587	0.468
A11_Hill	46	4	3.98	163–175	0.532	0.478	0.819	45	4	4.00	163–175	0.186	0.200	1.000
A16_M13	46	12	12.00	156–194	0.864	**0.652**	**0.000**	46	10	10.00	170–194	0.650	**0.587**	0.045
A18_M13	46	9	9.00	211–243	0.833	0.804	0.308	44	8	8.00	203–239	0.839	0.727	0.084
A19_T3	46	6	5.93	242–274	0.257	**0.152**	**0.010**	45	7	7.00	250–274	0.266	0.267	0.362
A20_Neo	45	2	2.00	178–182	0.022	0.022	–	46	5	4.98	164–182	0.323	0.304	0.344
A22_Neo	46	5	4.98	214–239	0.338	**0.217**	**0.006**	45	5	5.00	214–243	0.428	0.422	0.927
A30_T3	45	7	7.00	245–269	0.784	0.578	0.023	44	7	7.00	245–269	0.840	**0.545**	**0.000**
A31_T3	46	7	6.96	318–374	0.331	0.304	0.056	45	11	11.00	314–386	0.306	0.244	**0.004**
A33_M13	46	12	11.90	233–277	0.729	**0.435**	**0.000**	42	15	15.00	241–297	0.884	**0.643**	**0.001**
A34_M13	46	7	6.96	297–321	0.699	0.674	0.058	44	6	6.00	297–317	0.636	0.682	0.415
A37_Neo	46	21	20.52	265–489	0.844	0.739	**0.004**	44	25	25.00	289–441	0.929	0.886	0.465
A39_Hill	46	12	12.00	356–408	0.739	0.674	0.092	46	12	12.00	364–416	0.685	0.696	0.144
A43_T3	46	15	14.89	309–369	0.754	0.630	0.374	45	9	9.00	331–359	0.754	0.689	0.556
B01_Hill	46	13	13.00	129–181	0.735	0.717	0.391	46	14	14.00	129–213	0.838	0.826	0.463
B03_T3	46	6	5.91	147–161	0.394	0.435	1.000	44	4	4.00	151–161	0.226	0.250	1.000
B07_M13	46	9	9.00	163–181	0.602	**0.435**	0.081	46	9	9.00	165–187	0.488	**0.326**	0.019
B12_T3	45	11	10.96	346–398	0.741	**0.467**	**0.000**	44	10	10.00	346–398	0.653	**0.409**	**0.001**
B15_T3	46	4	4.00	170–178	0.478	0.500	0.562	46	4	4.00	170–178	0.382	0.435	0.839
B19_T3	46	13	12.93	242–290	0.806	0.783	0.677	45	19	19.00	242–320	0.799	0.867	0.206
B28_Neo	46	4	4.00	226–238	0.536	0.391	0.116	46	4	4.00	226–235	0.507	0.565	0.213
B29_Neo	46	9	8.87	262–281	0.738	0.783	0.894	44	5	5.00	265–278	0.509	0.545	0.612
B30_Neo	46	6	6.00	154–169	0.742	0.783	0.250	45	7	7.00	151–169	0.693	**0.622**	0.222
B33_M13	46	5	4.96	120–135	0.354	0.413	0.690	45	6	6.00	120–135	0.463	0.533	0.896
B36_Hill	41	2	2.00	384–393	0.048	**0.000**	0.013	41	3	3.00	390–396	0.291	**0.098**	**0.000**
	Celtic Sea							Scotian Shelf						

Locus name	*n*	*a*	R_S	*as*	H_E	H_O	HW	*n*	*a*	R_S	*as*	H_E	H_O	HW
B38_Neo	46	3	3.00	409–415	0.365	0.370	1.000	46	4	4.00	388–415	0.065	0.065	1.000
C01_M13	46	14	13.65	127–195	0.844	**0.543**	**0.000**	39	8	8.00	115–151	0.833	**0.410**	**0.000**
C08_T3	46	9	8.98	149–185	0.757	0.761	0.765	45	10	10.00	145–181	0.855	0.800	0.041
C13_Neo	46	5	5.00	158–173	0.276	0.239	0.300	46	6	6.00	158–176	0.242	0.261	1.000
C14_Hill	45	6	6.00	146–166	0.751	**0.644**	0.062	43	4	4.00	146–158	0.631	0.535	0.425
C15_Hill	45	18	18.00	171–205	0.903	**0.667**	**0.000**	46	15	14.91	169–187	0.796	0.761	0.178
C17_M13	46	8	8.00	154–190	0.749	**0.609**	0.059	46	7	7.00	154–182	0.788	0.609	0.083
C20_Neo	45	4	3.96	227–236	0.188	**0.089**	**0.001**	43	5	5.00	227–239	0.276	**0.140**	**0.000**
C22_Hill	46	9	9.00	237–273	0.785	0.739	0.624	46	9	9.00	225–273	0.799	0.848	0.522
C28_Neo	46	7	7.00	258–282	0.580	0.543	0.338	46	7	7.00	255–279	0.480	0.500	0.951
C30_Neo	46	2	2.00	337–340	0.505	0.435	0.389	46	3	3.00	337–343	0.473	0.370	0.229
C31_Neo	45	4	4.00	332–341	0.516	0.556	1.000	46	3	3.00	332–338	0.389	0.391	0.657
C35_M13	44	9	9.00	241–348	0.605	0.591	0.972	46	6	6.00	233–281	0.329	0.326	0.832
C36_Hill	46	6	6.00	319–352	0.347	0.326	0.827	46	7	7.00	325–349	0.260	0.283	1.000
C40_M13	46	2	2.00	276–277	0.410	0.435	1.000	46	2	2.00	276–277	0.161	0.174	1.000
C42_M13	45	2	2.00	354–357	0.481	0.511	0.756	46	8	7.96	345–358	0.711	0.674	0.754
D05_Hill	45	8	8.00	230–262	0.478	0.489	0.526	46	10	9.89	230–270	0.659	0.717	0.984
D10_Neo	46	2	2.00	308–312	0.436	0.500	0.498	46	2	2.00	308–312	0.452	0.370	0.318
D12_Hill	46	7	6.98	259–279	0.711	0.717	0.055	45	8	8.00	251–291	0.369	0.333	0.049
D14_Hill	46	12	12.00	228–276	0.792	0.739	0.374	46	5	5.00	228–244	0.557	0.565	0.272
D15_Hill	46	3	2.98	244–252	0.144	0.152	1.000	45	4	4.00	244–256	0.169	0.178	1.000
D16_T3	44	4	4.00	424–436	0.340	0.341	0.504	45	4	4.00	424–436	0.207	0.222	1.000
D21_Neo	46	4	3.96	337–343	0.125	0.130	1.000	45	5	5.00	341–389	0.297	0.244	0.128
D30_M13	46	4	4.00	321–333	0.521	0.565	0.939	45	5	5.00	317–333	0.602	0.467	0.043
D35_M13	45	12	11.93	316–360	0.847	**0.667**	**0.002**	44	10	10.00	316–360	0.709	**0.455**	**0.000**
D37_T3	46	4	4.00	321–333	0.572	0.522	0.372	46	5	5.00	313–333	**0.567**	0.870	**0.000**
D43_T3	45	8	8.00	264–292	0.831	**0.444**	**0.000**	41	8	8.00	260–288	0.814	**0.341**	**0.000**
D46_Neo	46	4	3.96	143–155	0.164	0.174	1.000	45	4	4.00	139–151	0.406	0.444	0.337
Average across loci		**7.40**	**7.37**		**0.57**	**0.50**			**7.49**	**7.48**		**0.54**	**0.48**	

Table A3. Loci that exhibited a different repeat motif than initially identified from the raw sequence.

Locus	Expected motif	Detected motif
A16_M13	tetra	di
A22_Neo	tetra	di-tetra
A43_T3	tetra	di
B29_Neo	tri	mono
C15_Hill	tetra	mono
C40_M13	tri	mono
C42_M13	tri	mono
D05_Hill	tetra	di
D21_Neo	tetra	di
D35_M13	tetra	di-tetra

Table A4. Loci showing signs of presence of null alleles.

Celtic Sea	Scotian Shelf
A04_Neo	A04_Neo
A16_M13	A30_T3
A19_T3	A33_M13
A22_Neo	B07_M13
A30_T3	B12_T3
A33_M13	B36_Hill
B07_M13	C01_M13
B12_T3	C17_M13
C01_M13	C20_Neo
C15_Hill	D35_M13
C20_Neo	D43_T3
D35_M13	
D43_T3	